Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 14 (2006) 537–543
Bioorganic & Medicinal Chemistry

Reduction of nonspecific binding proteins to self-assembled monolayer on gold surface

Minoru Furuya, Masayuki Haramura and Akito Tanaka*

Chemistry Department, Reverse Proteomics Research Institute Co., Ltd, 2-6-7 Kazusa-Kamatari, Chiba 292-0818, Japan

Received 15 August 2005; revised 9 September 2005; accepted 9 September 2005
Available online 28 November 2005

Abstract—We developed a gold coated glass chip bearing a poly(ethyleneglycol) (PEG) type compound as hydrophilic spacer for surface plasmon resonance studies, which enabled adequate estimation of K_d value between FK506 and FKBP12 not only using purified FKBP12 (K_d = 22 nM) but also using *Escherichia coli* lysate expressing FKBP12 (K_d = 15 nM). These results indicated effectiveness of the PEG spacer for reduction of nonspecific interactions. Chemical stability and simple surface-structure of the novel chip are also attractive.

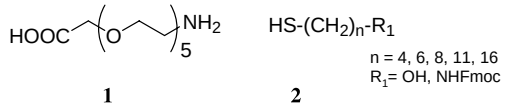
1. Introduction

Self-assembled monolayers (SAMs) on gold surfaces are important for investigation of the intermolecular interactions, for example, protein–protein, protein–small molecule, and so forth, using a surface plasmon resonance (SPR)[1–4] and a quartz crystal microbalance (QCM).[5–8] Reduction of nonspecific protein binding is especially important in these techniques, because interactions on SAMs are usually estimated from the total resonances which include both of specific and nonspecific interactions. We reported in a previous paper that amount of nonspecific absorption on affinity resins has a linear relationship with hydrophobicity of immobilized ligands, and they could be reduced by introduction of a new poly(ethyleneglycol) (PEG) type hydrophilic spacer (**1**, Scheme 1) between ligands and solid surfaces.[9] Prime and Whitesides also reported that nonspecific interactions of four commercial available proteins, fibrinogen, lysozyme, pyruvate kinase, and RNAse A, to SAMs on gold surface depend on hydrophilic properties at the surface, and introduction of other PEG spacers could reduce them.[10] There was, however, no report that addressed nonspecific interactions of proteins to SAMs on gold surfaces using lysate obtained from organs or cell lines, while investigation using such crude mixture is wealthy since it is usually difficult to obtain pure proteins.

SAMs used in SPR or QCM are basically constructed by alkanethiolates (**2**, Scheme 1), which consist of a thiol group, a hydrophobic alkane moiety, and a terminal functional group (R_1). The thiol group immediately binds to gold surface, and hydrophobic alkane chains construct a hydrophobic layer which stabilize the structure of SAMs in aqueous mixtures.[10] Terminal groups are used to immobilize compounds through hydrophilic spacer moieties. The stability of SAMs usually depends on lengths of the alkane moiety (n). It was reported that eleven and more length methylene was needed in ω-substituted alkanethiolates to make stable SAMs in aqueous solutions.[10–12] Hydrophilic spacers were widely used for increase of hydrophilicity and flexibility of compounds at their surfaces, and were thought to be important for reduction of the nonspecific absorption. Dextran, a sugar derivative, was widely used for this purpose.[13] However, it is usually not stable under most of synthetic conditions so that immobilization of ligands was carried out in aqueous solutions with excess amount of ligands and coupling reagents, which enforce exhaustion of the costly ligands. Low solubility of ligands, especially synthetic compounds, in aqueous solutions sometimes makes it difficult to immobilize sufficient amount of ligands on the SAMs.[14] The thick (~100 nm) hydrogel layer of dextran sometime complicates analysis on the results.[15,16] Thus, development of new chemically stable and hydrophilic spacer, enough to reduce the nonspecific binding, is desired.

Keywords: Nonspecific binding proteins; Surface plasmon resonance; FK506; FKBP12.
* Corresponding author. Tel.: +81 438 52 3990; fax: +81 438 52 3986; e-mail: tanaka-a@reprori.jp

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.09.030



1 2

Scheme 1.

We here report that (1) synthesis of gold materials bearing several alkanethiols (**2**) and structure–property relationships on amount of binding proteins to them after being mixed with lysate from rat brain, (2) design of novel gold material bearing **1** based on the relationship, (3) reduction of nonspecific binding proteins by introduction of **1** maintaining specific binding, and (4) ability of a gold-coated glass chip bearing **1** to analyze the interaction between FK506 and FKBP12 by SPR not only using purified FKBP12 but also using *Escherichia coli* lysate expressing FKBP12.

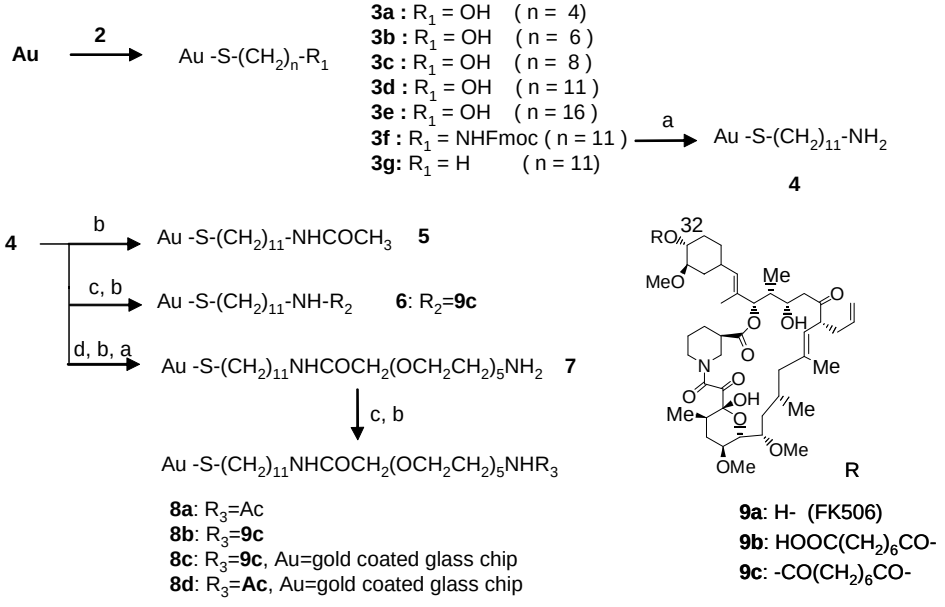
2. Chemistry

Structures and synthesis of all materials used in this work are shown in Scheme 2. Immobilizations of alkanethiolates (**2**) on gold surfaces were carried out in ethanol at rt to afford SAMs (**3a**–**g**) on gold surface. Gold foils and gold coated glass chip were used as a starting material for synthesis of **3**, **5**, **6**, **8a**,**b** and for that of **8c**,**d**, respectively. SAMs bearing amines at terminal moiety (**4** and **7**) were synthesized via Fmoc-protected intermediates. Acetylation of amines on SAMs was carried out by mixing of acetic acid with EDC·HCl and HOBt because that by acetic anhydride disordered the stable SAMs.[19] Compound **9b** was designed based on the crystal structure of FK506 with FKBP12, which exhibited that hydroxy group at the 32 position was suitable for addition of linker moiety because it was not involved in the interaction.[9] Immobilization of FK506 derivative (**9b**) and acetylation on gold coated glass chips were performed using Biacore 3000 with Surface prep unit (Biacore).

3. Results and discussion

3.1. Nonspecific binding proteins on gold substrates

We first assessed the critical length of methylene (*n*) in alkanethiols (**2**) for construction of stable SAMs on gold surface by analysis of binding proteins using SDS–polyacrylamide gel electrophoresis (SDS–PAGE) after being mixed with rat brain lysate because previous studies observed the relationships only using a mixture of isolated proteins.[10–12] Lysate obtained from rat brains includes thousands of proteins as shown in Figure 1A (lane 2) and was thought to be suitable for assessment of nonspecific binding proteins on them. Gold materials bearing ω-hydroxyalkylthiol with several lengths of methylene (**3a**–**e**) were mixed with the lysate. After extensive washes with lysate buffer (25 mM Tris–HCl (pH 7.4), 0.25 M sucrose), binding proteins were completely eluted by a SDS sample buffer solution (4% (w/v)—SDS, 20% (v/v)—glycerol, 0.01% (w/v)—BB, 10% (v/v)—2-ME, and 0.125 M Tris, pH 6.8) and analyzed by SDS–PAGE (Fig. 1A). There were several nonspecific binding proteins on naked gold foil (lane 3). Mass spectrometry analysis after in-gel digestion on the largest protein showed that was tubulin, one of a representative nonspecific binding protein.[9] There were large amounts of binding proteins on gold foils bearing ω-hydroxybutanethiol (*n* = 4, **3a**, lane 4) more than on naked gold foil. The reason for this increase was thought that the surface had disordered SAMs and those proteins could nonspecifically bind to the hydrophobic moieties of ω-hydroxybutanethiol such as butane moiety. Amounts of these nonspecific binding proteins were



Scheme 2. Reagents: (a) 20% piperidine in CH_3CN; (b) AcOH, WSC, HOBt/DMF; (c) **9b**, WSC, HOBt/DMF; (d) *N*-Fmoc-**1**, PyBOP, $^{i}Pr_2NEt$/CH_3CN.

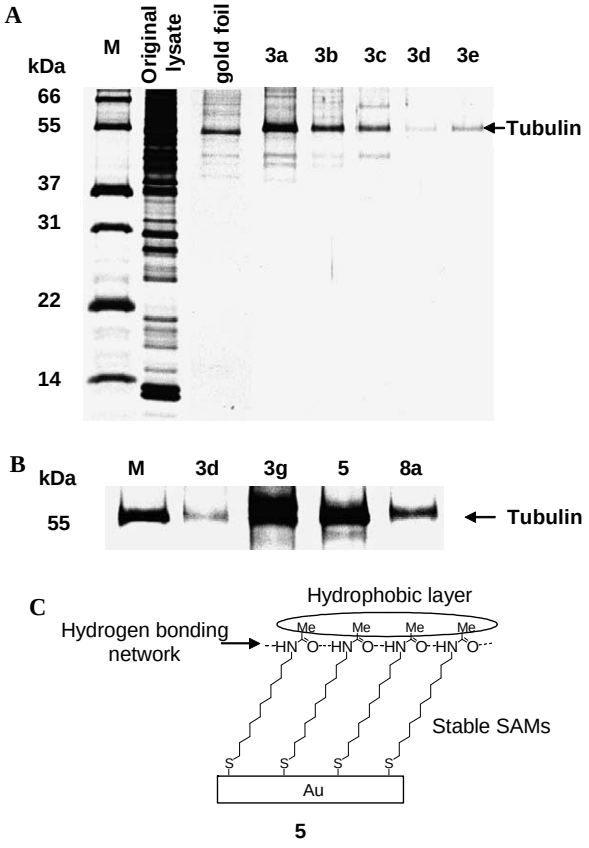


Figure 1. (A) Binding proteins on gold foil and SAMs which consist of different lengths of methylene (**2a**–**e**). (B) Binding proteins to gold foil surface with various terminal functional groups. (C) Presumable structure of **5** for the explanation of the large amount of tubulin on it.

decreased by increase in the number of methylene chains (n = 6, 8, 11, and 16, **3b**–**e**, lanes 4–8), and looks like it reached a minimum level at n = 11 (**3d**), which indicated that stable SAMs were constructed near the eleven length of methylene chain. This result is consistent with those of the previous works. Thus, we fixed the length of the methylene chain of alkanethiols to be eleven in this study.

The next amount of tubulin bound on gold materials bearing ω-substituted n-undecanethiols with four kinds of functional groups, hydroxyl (**3d**), methyl (**3g**), acetylamino (**5**), and acetylamino with the PEG spacer (**8a**), was analyzed (Fig. 1B). These materials were thought to have a similar SAM structure, so these differences in the amount could due to properties of the terminal moieties.The amount of tubulin very much increased by replacement of hydroxy (**3d**) with hydrogen (**3g**) (lanes 2 and 3). This result is consistent with that of a previous study obtained on affinity resins[9] in which increase of hydrophobicity resulted in induction of nonspecific protein bindings. Interestingly, the amount of tubulin on gold material with acetylamino (**5**) was more than that on **3d** (lane 4) and similar to that on a methyl derivative (**3g**). This result indicated that simple hydrophobicity could not account for nonspecific binding proteins alone because acetylamino moiety has equivalent hydrophilicity to that of hydroxyl and more hydrophilicity than hydrogen atom based (e.g., the π values of NHAc, OH, and hydrogen are −0.97, −0.97, and 0.00, respectively).[20] We thought the reason for this unexpected large amount of tubulin on **5** was that stable hydrogen bond network between closely adjoined acetylamino moieties was formed at the surface as illustrated in Figure 1C, which resulted in direction of the methyl group toward aqueous phase and effected construction of a kind of hydrophobic methyl layer.

Introduction of **1** between the aminoacetyl group and SAMs (**8a**) resulted in much reduction of binding of tubulin (lane 5). This result supported the above speculation because insertion of **1** could introduce flexibility of the acetyl moiety with increase of hydrophilicity.

3.2. Reduction of nonspecific binding proteins on gold surface by introduction of 1

Introduction of **1** successfully reduced nonspecific protein binding to SAMs on gold foils (Fig. 1B), so we next compared specific binding proteins with nonspecific ones either on gold foils or gold coated glass chips with or without **1**. We adopted FK506 as a bait compound for estimation of specific binding protein. FK506 is an immunosuppressive drug that targets FKBP12, FK506 binding protein, with a K_d of 0.4 nM.[21] FKBP12 was originally isolated FK506-affinity resins, and structure and functions of the complex of FK506 and FKBP12 have been well characterized at molecular level by Schreiber and co-workers.[22–24] Since the molecular weight of FKBP12 (12 kDa) differs significantly from that of tubulin (50 kDa), it is easy to observe and compare the amounts of specific and nonspecific protein binding. It is known that the lysate obtained from rat brains includes FKBP12.[9]

Gold material bearing FK506 without **1** on SAMs (**6**) captured a small amount of FKBP12 with a large amount of tubulin and other nonspecific ones (Fig. 2, lane 3). Insertion of **1** between SAMs and FK506 (**8b**) resulted in improvement of selectivity; that is, the

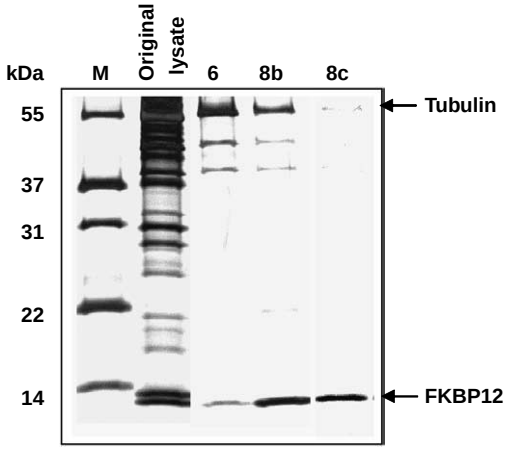


Figure 2. Binding proteins on gold surface bearing FK506 at their terminals without (**6**) or with the PEG spacer (**8b** and **c**) after being mixed with lysate obtained from rat brain.

amount of FKBP12 was much increased, while absorption of the nonspecific protein binding decreased as expected (lane 4). This result indicated that insertion of **1** could afford appropriate environment for binding of protein with the ligand.

Chemical stability of **1** is attractive for us under synthetic conditions, for example, **1** was quite stable under synthetic conditions used in Scheme 2, since the widely used hydrophilic spacer, dextran, in SPR studies is not chemically stable and is usually used only in aqueous reaction mixtures. Immobilization of ligands on such dextran chips requires excess amount of ligands and coupling reagents, which enforce exhaustion of the ligands. Low solubility of ligands in aqueous solutions sometimes makes it difficult to immobilize sufficient amount of ligands on the chip.[14] Its putative monolayer structure can be attractive as well, since the thick structure of dextran sometimes complicated the analysis of SPR results.[15,16] Therefore, use of **1** is thought to be effective for analysis of the intermolecular interactions on gold surfaces such as SPR and QCM analysis.

SAMs are usually constructed on gold coated glass chip for SPR and QCM studies, not on gold foils. Thus, we applied **1** on gold coated glass chips as well. Gold coated glass chip bearing FK506 through **1** could capture a large amount of FKBP12 with little amount of nonspecific ones from the complicated mixture of proteins (lane 5). This difference could have arisen from the fact that structure of SAMs on the glass (**8c**) was more regulated and stable than that on gold foil (**8b**). These results indicated that the use of **8c** could make it possible to analyze the interaction between immobilized FK506 on the gold surface and FKBP12 specifically, not only using purified FKBP12 but also using a more complicated mixture of proteins such as *E. coli* lysate expressing FKBP12 without purifications.

3.3. Application of 1 to SPR study

Analysis of the interaction between FK506 and FKBP12 on gold coated glass chips bearing **1** by a Biacore3000 apparatus was carried out (Fig. 3). At first, binding of FKBP12 on acetyl capping gold material (**8d**) using purified FKBP12 was measured (Fig. 3A), which exhibited that nonspecific absorption of FKBP12 was little. Analysis of the interaction of FKBP12 and immobilized FK506 using **8c** and purified FKBP12 solution showed a good model of a 1:1 complex with K_d = 22 nM (Fig. 3B), which was almost 50 times weaker than the K_d value obtained from a conventional method (0.4 nM). This difference could be caused by immobilization of ligand to surface, resulting in limited flexibility of FK506 on the surface and hindrance effects from the solid surface. The response at equilibrium was not observed in the sensorgram, which showed that association and dissociation between FK506 on the chip and FKBP12 were slow.

Next we carried out SPR analysis using *E. coli* lysate expressing FKBP12 (Fig. 3C). Use of such crude lysate, as shown in Figure 3E, has been rarely tried in SPR studies because it was thought estimation of specific interactions should be confused by the signals from nonspecific ones. While a lot of SPR signals were observed on the acetyl capping gold material (**8d**), the result in Figure 2 indicates that these signals occurred due to some hydrophobic interactions of hydrophobic compounds included in the crude mixture, for instance, fatty acids, or other small molecules, with the SAMs, no but

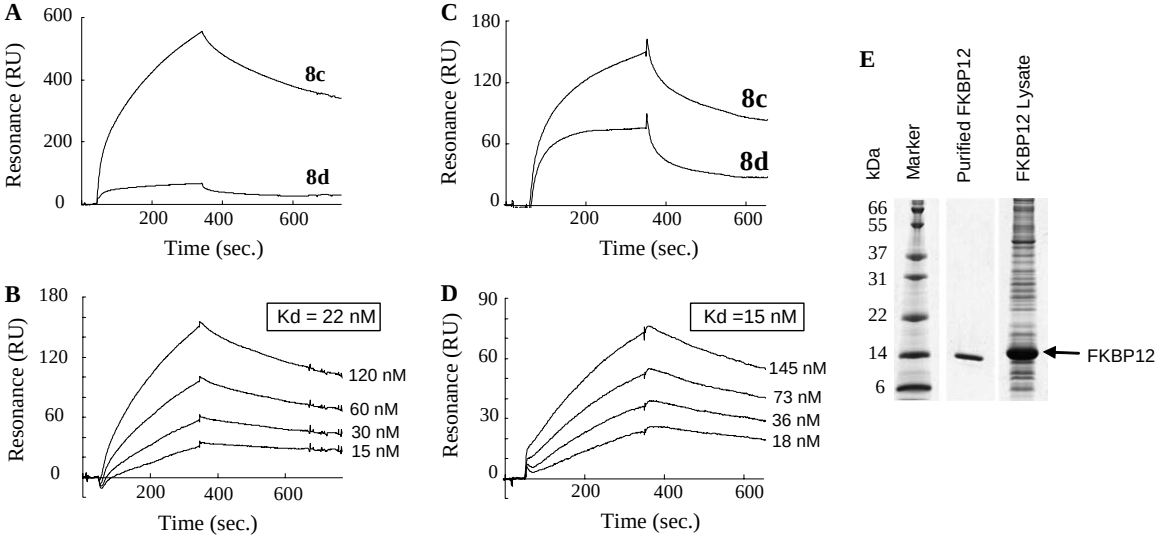


Figure 3. (A) Binding of purified FKBP12 on **8c** and **d**. Purified FKBP12 solution (950 nM) in HBS-EP buffer was injected for 5 min. HBS-EP buffer was flown through the other period of the analysis. The amount of protein was exhibited as a resonance value (RU).[26] (B) Binding of purified FKBP12 on **8c** at various concentrations. All SPR signals of FKBP12 solutions (15–120 nM) were reduced to that of **8d** as a control cell. Kinetic study was performed with BIA evaluation software (Ver. 4.1; Biacore). (C) Binding of *Escherichia coli* lysate expressing FKBP12 on **8c** and **d**. The *E. coli* lysate including 145 nM of FKBP12 in HBS-EP buffer was injected for 5 min. (D) Binding of FKBP12 on **8c** at various concentrations of the lysate. All SPR signals of each lysate (FKBP12; 18–145 nM) were reduced to that of **8d** as a control cell. The *E. coli* lysates HBS-EP buffer were flown for 5 min. (E) Purity of FKBP12 and *E. coli* lysate expressing FKBP12 used in this work (CBB stain); lane 1: marker, lane 2: purified FKBP12, and lane 3: *E. coli* lysate expressing FKBP12.

nonspecific interactions of proteins. These background signals should be counteracted by subtraction between **8c** and **d** since they may be constant on both.[25] Thus, we calculated the K_d value from these SPR signals, which resulted in a good model of 1:1 complex as well, with K_d = 15 nM (Fig. 3D), which was almost same as that using purified FKBP12. This consistency substantiated the effectiveness of **1** as hydrophilic spacer in researches of intermolecular interactions on gold surfaces even using a crude mixture of protein such as lysate.

4. Conclusions

Self-assembled monolayers (SAMs) on gold surfaces are important for investigation of the intermolecular interactions. Reduction of nonspecific protein binding on them is important in researches using SPR and QCM because interactions are usually estimated from the total resonance values including both of specific and nonspecific interactions. In order to circumvent this problem, analysis of nonspecific binding proteins on SAMs on gold materials after being mixed with lysates was performed using SDS–PAGE. Analysis by SDS–PAGE was vital in this study since these methods could afford quantitative and individual information on each protein bound on the surfaces (Fig. 1). These studies showed that amount of nonspecific binding protein could be reduced by introduction of PEG type spacer (**1**).[9] Insertion of **1** between SAMs and FK506 on a gold coated glass chip (**8c**) resulted in increase of the specific interaction with FKBP12 whilst reducing the nonspecific ones (Fig. 2). Based on this result, we analyzed the interaction of FK506 and FKBP12 on **8c** by the SPR method using purified FKBP12 and *E. coli* lysate expressing FKBP12 (Fig. 3). Analysis using this lysate showed a good model of 1:1 complex, with K_d = 15 nM, which was almost same as that using purified FKBP12 (K_d = 22 nM). This result exhibited effectiveness of **1** as a hydrophilic spacer in researches of intermolecular interactions on gold surfaces as well. We have not succeeded in estimating the interaction using lysate from rat brain (data not shown). We thought the reason for this difference is that lysate obtained from rat brain included a large amount of hydrophobic compounds such as fatty acids, etc., more than that from *E. coli*. But we believe that investigation using the *E. coli* lysate is wealthy. Chemical stability and simple surface-structure of this novel chip are also attractive, compared to the dextran chips.

5. Experimental

The following abbreviations were used: CH_3CN, acetonitrile; AcOH, acetic acid; BB, bromophenol blue; buffer A, an aqueous solution of 25 mM Tris–HCl (pH 7.4) and 0.25 M sucrose; DMF, dimethylformamide; EDC·HCl, *N*-ethyl-*N*′-(3-dimethylamino-propyl)carbodiimide hydrochloride; EtOH, ethanol; FK506, immunosuppressant tacrolimus; HOBt, 1-hydroxy-benzotriazole; 2-ME, 2-mercaptoethanol; PEG, poly(ethyleneglycol); PyBOP, bromotris(pyrrolidino)phosphonium hexafluorophosphate; $^{i}Pr_2NEt$, diisopropylethylamine; rt, room temperature; SDS, sodium dodecyl sulfate.

5.1. Materials

All reagents were used as received. $HS(CH_2)_{10}CH_3$ was obtained from Tokyo Kasei Kogyo Co., Ltd. $HS(CH_2)_4OH$, $HS(CH_2)_{16}OH$, piperidine, $^{i}Pr_2NEt$, and AcOH were purchased from Aldrich. $HS(CH_2)_6OH$, $HS(CH_2)_8OH$, $HS(CH_2)_{11}OH$, and $HS(CH_2)_{11}NHFmoc$ were obtained from Dojindo Laboratories. EDC·HCl and HOBt were obtained from Watanabe Chemical Industries, Ltd. HOBt was obtained from Peptide Institute, Inc. Gold foil (100 × 100 × 0.01 mm, purity 99.9% up) was purchased from High Purity Chemicals. SIA chips and HBS-EP buffer were purchased from Biacore AB (Uppsala, Sweden). Compounds *N*-Fmoc protected **1** and **9b** were prepared as described previously.[12]

5.2. Preparation of SAMs on gold foil (3a)

All gold foil was cut into squares (1.0 × 1.0 cm). A gold foil was treated with freshly prepared piranha solution (3:1 concentrated H_2SO_4/30% H_2O_2) for a few hours at rt, rinsed with thoroughly deionized water and EtOH, and dried under a stream of N_2. This gold foil was immersed for at least 16 h in 1.5 mM $HS(CH_2)_4OH$ in EtOH solution. After this, gold foil was rinsed with EtOH and dried under a stream of N_2.

Other SAMs on gold surface (**3b**–**g**) were prepared in a similar manner. Compounds **3a**–**e** and **g** were stored at 4 °C in 20% aqueous EtOH solution until binding assay.

Caution: piranha solution reacts violently with organic materials and must be handled with extreme care.

5.3. Au-S$(CH_2)_{11}NH_2$ (4)

Compound **3f** was immersed in 1 mL of a mixture of piperidine and CH_3CN (1:4), and shaken calmly for 30 min at rt. After removal of this solution, gold foil was washed with CH_3CN and dried under a stream of N_2 to give **4**. The amount of amino moiety on **4** was estimated to be 1.0 pmol/mm^2 using LC/MS analysis of Fmoc deprotection product as follows: after washing with CH_3CN, the gold materials bearing Fmoc derivatives were treated with 1 mL of 20% piperidine in CH_3CN for 0.5 h under shaking. The piperidine solution was collected and the gold foil was washed with 1 mL CH_3CN. The CH_3CN solution was collected and mixed with the piperidine solution. The mixture was evaporated to dryness. The residue was dried under reduced pressure at 50 °C for 1 h and cooled to rt. CH_3CN (100 μL) and 100 μL of H_2O were added to dissolve the residue. After filtration, the solution was applied on LC/MS. The LC/MS analysis was performed with Agilent 1100 series. The condition of HPLC was as follows: column, reverse-phase (Capcell Pak ACR C18, 2.0 mm id × 35 mm; Shiseido); mobile phase, 0.1% aqueous trifluoroacetic acid solution/CH_3CN, linear gradient from 95/5 to 5/95 for 4 min duration. The detection was performed using mass spectrometry by positive ion signal

of *N*-fluorenyl methyl piperidine (Fmoc deprotection product); 264 [M+H].[17] The amount of Fmoc moiety was calculated from an accumulated area of the mass peak and corrected with a calibration curve of a standard sample.

5.4. Au-S$(CH_2)_{11}$-NHAc (5)

Compound **4** was immersed in 1 mL of a mixture of AcOH (10 mM), EDC·HCl (10 mM), and HOBt (10 mM) in CH_3CN, and shaken calmly at rt for 5 h. After removal of the mixture, gold foil was washed with CH_3CN to give **5**.

Au-S$(CH_2)_{11}$-NHCO-$CH_2(OCH_2CH_2)_5$NHAc (**8a**) was prepared in a similar manner above. Compounds **5** and **8a** were stored at 4 °C in 20% aqueous EtOH solution until binding assay.

5.5. Au-S$(CH_2)_{11}$NHCO$(CH_2)_6$CO-FK506 (6)

Compound **4** was immersed in 1 mL of a mixture of EDC·HCl (10 mM), HOBt (10 mM), and **9b** (10 mM) in DMF, and was shaken calmly for 15 h at rt. After removal of the mixture, gold foil was washed with DMF. The resulting gold foil was immersed in 1 mL of a mixture of AcOH (10 mM), EDC·HCl (10 mM), and HOBt (10 mM) in DMF, and shaken calmly at rt for 5 h. After removal of the mixture, the gold foil was washed with DMF to give **6**.

Au-S$(CH_2)_{11}$-NHCO-$CH_2(OCH_2CH_2)_5$NHCO$(CH_2)_6$CO-FK506 (**8b**) was prepared from **7** in a similar manner.

Compounds **6** and **8b** were stored at 4 °C in 20% aqueous EtOH solution until binding assay.

5.6. Au-S$(CH_2)_{11}$NHCO-$CH_2(OCH_2CH_2)_5NH_2$ (7)

Compound **4** was immersed in 1 mL of a mixture of PyBOP (50 mM), $^{i}Pr_2NEt$ (100 mM), and FmocNH$(CH_2CH_2O)_5CH_2COOH$ (*N*-Fmoc-**1**, 50 mM) in CH_3CN, and shaken calmly for 15 h at rt. After removal of the mixture, gold foil was washed with CH_3CN. The resulting gold foil was immersed in 1 mL of a mixture of AcOH (10 mM), EDC HCl (10 mM), and HOBt (10 mM) in CH_3CN, and shaken calmly at rt for 5 h. After removal of the mixture, the gold foil was washed with CH_3CN, and then gold foil was immersed into 1 mL of 20% piperidine in CH_3CN and shaken calmly for 30 min at rt. After removal of the mixture, the gold foil was washed with CH_3CN and dried under a stream of N_2 to afford **7**. The amount of amino moiety on **7** was estimated to be 0.75 pmol/mm^2 using LC/MS analysis of Fmoc deprotection product as mentioned above. Yield of the reaction from **4** to **7** was estimated as 75%.

5.7. Au(gold coated glass chip)-S$(CH_2)_{11}$NHCO-$CH_2(OCH_2CH_2)_5$NHCO$(CH_2)_6$CO-FK506 (8c)

SIA chip (Biacore AB, Uppsala, Sweden) was used as a gold substrate. And **8c** was prepared in the same manner with **8b**. Compound **8c** was stored at 4 °C in 20% aqueous EtOH solution until binding assay. The amounts of amino moiety of **4** on gold coated glass chip and **7** on gold coated glass chip were estimated to be 1.2 pmol/mm^2 and 1.08 pmol/mm^2, respectively, using LC/MS analysis of Fmoc deprotection product. Yield of the reaction from **4** to **7** on gold coated glass chip was estimated as 90%.

5.8. Preparation of rat brain lysate

Preparation of tissue extracts of rat brain. Fresh rat brain were homogenized (1:10, w/v) in buffer A. The homogenate was centrifuged at 9500 rpm for 10 min. After supernatant was separated, it was centrifuged at 50,000 rpm for 30 min again. The obtained supernatant was used for lysate and kept at −80 °C before use.

5.9. Binding assay on gold foils or gold coated glass chips

These surface modified gold foils or gold coated glass chips were washed with 1 mL buffer A three times at 4 °C before binding assay. The lysate as a protein mixture was diluted by buffer A and total protein concentration was prepared to about 7 mg/mL. This lysate was shaken calmly with surface modified gold foils or gold coated glass chips at 4 °C for 15 h to bind the nonspecific binding proteins or specific binding proteins. A typical lysate solution has a total volume of 1.0 mL, consisting of buffer A, and 0.5 mL of tissue extract. After incubation, the lysate solution was removed. The gold foils or gold coated glass chips were washed five times with 1.0 mL buffer A. The washed gold foils were then resuspended in 25 μL SDS sample buffer solution (Nacalai, sample buffer solution with 2-ME(2×) for SDS–PAGE, cat. 30566-22, including 4% (w/v)—SDS, 20% (v/v)—glycerol, 0.01% (w/v)—BB, 10% (v/v)—2-ME, and 0.125 M Tris, pH 6.8) and shaken at 25 °C for 10 min. The supernatant was subjected to SDS–PAGE followed by silver staining.

5.10. Surface plasmon resonance spectroscopy

The Biacore3000 instrument was used for all SPR study described in this paper. Our substrates on the glass chip were incorporated into the Biacore cassette using BIA SIA kit (Biacore). HBS-EP buffer (0.01 M HEPES, pH 7.4; 0.15 M NaCl; 3 mM EDTA; 0.005% polysorbate20: Biacore) was used through the SPR study. For SPR measurement, we used the KINJECT command. The data were collected at a flow rate of 40 μL/min for the protein solutions in HBS-EP buffer. Regeneration was performed with 10 μL of 0.5% SDS in HBS-EP buffer before the next sample injection. The data were analyzed by standard procedure using BIA evaluation software (Ver. 4.1, Biacore).

5.11. Immobilization on Biacore 3000 using surface prep unit

Compound **7** on gold coated glass chip assembled into Biacore cassette was docked in surface prep unit with

type 1 flow cell carrier on Biacore 3000. After washing the flow cell with CH_3CN (100 μL/min, 2 min), the mixture of **9b** (10 mM), EDC (10 mM), and HOBt (10 nM) in CH_3CN (900 μL) was injected for 900 min (1 μL/min). After washing with CH_3CN (100 μL/min, 2 min), the mixture of AcOH (10 mM), EDC (10 mM), and HOBt (10 nM) in CH_3CN (300 μL) was injected for 300 min (1 μL/min) for acetyl capping, resulting in the formation of **8c** on the flow cell. Only Ac capping procedure was used for **8d** on the flow cell as a control cell.

5.12. Preparation of *E. coli* lysate expressing FKBP12

FKBP12 was expressed as a His_6-fusion using the plasmid pDest 17 and Gateway Cloning system (Invitrigen). His_6-FKBP production was induced with IPTG in BL21 (DE3) cells. The lysate was harvested using standard conditions (Fig. 3E, lane 3). The dialysis against HBS-EP buffer was performed before SPR study.

5.13. Purification of human FKBP12

FKBP12 was purified from the lysate described above by metal ion affinity chromatography according to a previous report.[18] Cation exchange chromatography was then performed with Resource S (1 mL) column on AKTA 10S system (Amasham Biosciences). The protein fraction was eluted with a 0 to 0.8 M NaCl gradient (30 mL total volume) in 20 mM HEPES buffer (pH 7.0) at a flow rate of 1 mL/min. Fractions including FKBP12 were combined and used as FKBP12 sample (Fig. 3E, lane 2). The dialysis against HBS-EP buffer was performed before the SPR study.

Acknowledgments

We thank Dr. David Barrett (Fujisawa Pharmaceutical Co., Ltd.) for his critical reading of the manuscript. The financial support from NEDO (The New Energy and Industrial Technology Development Organization) is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.09.030.

References and notes

1. Svedhem, S.; Enander, K.; Karlsson, M.; Sjöbom, H.; Liedberg, B.; Löfås, S.; Mårtensson, L.-G.; Sjöstrand, S. E.; Svensson, S.; Carlsson, U.; Lundström, I. *Anal. Biochem.* **2001**, *296*, 188.
2. Cannon, M. J.; Myszka, D. G.; Bagnato, J. D.; Alpers, D. H.; West, F. G.; Grissom, C. B. *Anal. Biochem.* **2002**, *305*, 1.
3. Cairo, C. W.; Strzelec, A.; Murphy, R. M.; Kiessling, L. L. *Biochemistry* **2002**, *41*, 8620.
4. Cooper, M. A.; Fiorini, M. T.; Abell, C.; Williams, D. H. *Bioorg. Med. Chem.* **2002**, *8*, 2609.
5. Kastl, K.; Ross, M.; Gerke, V.; Steinem, C. *Biochemistry* **2002**, *41*, 10087.
6. Miura, Y.; Sasao, Y.; Dohi, H.; Nishida, Y.; Kobayashi, K. *Anal. Biochem.* **2003**, *310*, 27.
7. Kaiser, T.; Gudat, P.; Stock, W.; Pappert, G.; Grol, M.; Neumeier, D.; Luppa, P. D. *Anal. Biochem.* **2000**, *282*, 173.
8. Murata, M.; Gouda, C.; Yano, K.; Kuroki, S.; Suzutani, T.; Katayama, Y. *Anal. Sci.* **2003**, *19*, 1355.
9. Tamura, T.; Terada, T.; Tanaka, A. *Bioconjug. Chem.* **2003**, *14*, 1222.
10. Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714.
11. Zhong, C.-J.; Porter, M. D. *J. Electroanal. Chem.* **1997**, *425*, 147.
12. Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G. *J. Am. Chem. Soc.* **1989**, *111*, 21.
13. For instance, Biacore home page, http://www.biacore.com/home.lasso.
14. For example, it was difficult to immobilize sufficient amount of FK506 derivative (**9b**) on commercial available chip for SPR (CM5 chip, Biacore) because of its low solubility (data not shown).
15. Glaser, R. W. *Anal. Biochem.* **1993**, *213*, 152.
16. Sigal, G. B.; Bamda, C.; Barberis, A.; Strominger, J.; Whitesides, G. M. *Anal. Chem.* **1996**, *68*, 490.
17. Meienhofer, J.; Waki, M.; Heimer, E. P.; Lambros, T. J.; Markofske, R. C.; Chang, C. *Int. J. Pept. Protein Res.* **1979**, *13*, 35.
18. Bollini, S.; Herbst, J. J.; Gaughan, G. T.; Verdoorn, T. A.; Ditta, J.; Dubowchik, G. M.; Vinitsky, A. *J. Biomol. Screen.* **2002**, *7*, 526–530.
19. The amount of the fluorene derivative, which was produced in the deprotection of a Fmoc group, was reduced after treatment of various SAMs with a 20% mixture of acetic anhydride in CH_3CN (unpublished data).
20. Hansch, C.; Leo, A.; Unger, S. H.; Kim, K.-H.; Nikaitani, D.; Lien, E. J. *J. Med. Chem.* **1973**, *16*, 1207.
21. Siekierka, J. J.; Hung, S. H.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755.
22. Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758.
23. Liu, J.; Farmer, J. D.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807.
24. Duyne, G. D. V.; Karplus, R. F. P. A.; Schreiber, S. L.; Clardy, J. *Science* **1991**, *252*, 839.
25. SPR signals due to these interaction tend to reach a maximum level soon at equilibrium conditions, shown in Figure 3C, while that due to the nonspecific absorption gradually increased for a long time because nonspecific absorption of proteins on hydrophobic surfaces usually causes denaturing of them with generation of new hydrophobic surfaces.
26. BIAapplications Handbook, 1998, Biacore AB, Uppsala, Sweden.